morphogenic proteins (BMPs) have also been tested as an adjunct therapy to accelerate the healing of fractures. BMP is an expensive therapy and has not been approved by the FDA for fracture treatment, only for spinal fusion. Clearly, there is an unmet clinical need of a less expensive adjunct therapy for better clinical management of fracture non-union. As a water-soluble monomer isolated from *Salviae miltiorrhizae*, tanshinol has been proved to be an effective bone anabolic agent with a high therapeutic index. However, its high water solubility and lack of chemical stability, has impeded its clinical application. To address this issue, we have developed a tanshinol-loaded bone-targeting liposome formulation (Tan-BTL). The objective of this study was to examine the potential therapeutic effects of Tan-BTL on fracture repair in mice.
**Methods**: The bone-targeting liposome (BTL) was labelled by rhodamine B and was used for a hydroxyapatite affinity test *in vitro* and a bone tissue targeting test *in vivo*. The BTL was labelled by IRdye 800CW for the observation of its distribution and retention time in the fracture site of mice. Tan-BTL (equivalent tanshinol dose 5 mg/kg, local administration once/week) was used for the treatment of a delayed fracture healing mouse model (induced by daily administration of prednisone at 12 mg/kg for 64 days). Planar X ray image monitored the healing process of the model for 64 days and the callus was analysed by micro-CT at the 18th day after fracture.
**Results**: Tan-BTL demonstrated significant hydroxyapatite affinity *in vitro*. Histological analysis of the distal femur after local administration of the formulation reveals robust targeting and retention at the growth plate, the trabecular bone, the cortical bone, and bone lacuna. A near infrared imaging analysis further confirmed BTL could concentrate on the fracture site of mice and be retain there up to 20 days after local injection. When tested in a delayed fracture healing mouse model (induced by daily administration of prednisone at 12 mg/kg for 64 days), micro-CT and planar X-ray imaging analysis of the callus tissue suggests that Tan-BTL increased callus BV/TV by 54% in a femur fracture of glucocorticoid-treated mice at the 18th day and shortened the fracture healing time from >64 days to 42 days when compared to glucocorticoid-treated mice without treatment.
**Conclusion**: These results support BTL as a promising targeted drug delivery system for local delivery of low molecular weight bone anabolic agents. Specifically, the Tan-BTL formulation tested could be a simple, safe, and effective non-invasive strategy for the treatment of bone fracture non-union.
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### 231
### ANGIOPOIETIN-LIKE PROTEIN 2 INDUCES INTERLEUKIN-6 EXPRESSION IN THE MECHANISM UNDERLYING LIGAMENTUM FLAVUM HYPERTROPHY IN LUMBAR SPINAL CANAL STENOSIS PATIENTS

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**Introduction**: Chronic inflammation is thought to cause ligamentum flavum (LF) degeneration and hypertrophy in lumbar spinal canal stenosis (LSCS). Angiopoietin-like protein 2 (Angptl2) is highly expressed in hypertrophied LF. Angptl2 regulates interleukin-6 (IL-6) expression in various tissues, thus we investigated whether IL-6 is expressed in hypertrophied LF and, if so, whether Angptl2 induces IL-6 expression in LF fibroblasts.
**Patients and Methods**: LF tissue was obtained from LSCS patients and non-LSCS patients. Polymerase chain reaction (PCR) for Angptl2 and IL-6 genes, and immunohistochemistry for IL-6 protein were performed in LF tissue. Fibroblasts from LF tissue were used for *in vitro* experiments. After Angptl2 recombinant protein treatment, NF-κB activation and IL-6 expression in LF fibroblasts were investigated by immunocytochemistry, PCR, and enzyme-linked immunosorbent assay.
**Results**: IL-6 mRNA expression was increased in hypertrophied LF tissue from LSCS patients and positively correlated with LF thickness and Angptl2 mRNA expression. IL-6 protein was highly expressed in LF fibroblasts in hypertrophied LF tissue. *In vitro* experiments demonstrated Angptl2 stimulation promoted NF-κB nuclear translocation and induced IL-6 expression and secretion in LF fibroblasts.
**Discussion and Conclusion**: This study provides evidence that Angptl2 could be a key molecule causing and promoting inflammation in LF tissue by activating IL-6 expression. IL-6 mRNA expression and IL-6-expressed fibroblasts were increased in hypertrophied LF compared with non-hypertrophied LF. Also, the expression was positively correlated with LF thickness and Angptl2 expression. Our *in vitro* experiments show that Angptl2 was able to elevate IL-6 expression via integrin α5β1/NF-κB signalling in LF fibroblasts. Angptl2 could promote inflammation in LF tissue by increasing IL-6 expression and secretion, resulting in LF degeneration and hypertrophy in LSCS patients. Anti-Angptl2 treatment could serve as a target in novel strategies for preventing LSCS and treating it.
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### 246
### MESENCHYMAL STEM CELLS PROMOTE VASCULOGENIC MIMICRY IN PROSTATE CANCER THROUGH SDF-1/ CXCR4 AXIS AND PI3K/Akt PATHWAY

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**Background**: Prostate cancer frequently metastasizes to the bone and the interaction between cancer cells and the bone microenvironment has proven to be crucial in the establishment of new metastases. Mesenchymal stromal cells (MSCs) in bone marrow may enhance tumour metastasis through secreting various cytokines that can regulate the behaviour of neighbouring cells. The term vasculogenic mimicry (VM) refers to the unique capability of aggressive tumour cells to mimic the pattern of embryonic vasculogenic networks. VM has been described in prostate cancer and some other highly aggressive tumours and is associated with tumour cell migration and invasion. However, the relationship between MSCs in their native bone marrow microenvironment and VM formation is not clear. Here we investigated the possible role of MSCs in VM by Prostate cancer cell lines, focusing primarily on the SDF-1/CXCR4 Axes and PI3K/Akt pathway.
**Subjects and Methods**: We studied the underlying mechanisms of VM in prostate cancer via the 3D culture system *in vitro* of PC-3 cells, expression of SDF-1, CXCR4, Akt, p-Akt and VE-cadherin proteins/mRNAs determined by ELISA, immunofluorescence, western blotting, and qRT-PCR, respectively.
**Results**: In this study, we show the effects of hMSCs on cancer cells are mediated through a secreted factor(s). In addition to enhanced proliferation when in co-culture with hMSCs, PC-3 cells were found to have increased the VM formation and CXCR4 expression *in vitro*. In addition, knocking down of CXCR4 using RNA interference or inhibition of CXCR4 function by an antagonist AMD3100 blocked hMSC-induced VM formations of PC-3 cells. Furthermore, hMSCs increased phosphorylation of Akt. Additionally, blocking PI3K/Akt Pathway using a PI3K inhibitor LY294002 decreased hMSCs induced VM formations of PC-3 cells. Under *in vivo* conditions, tumour growth and VM formation was promoted by MSCs in nude mice.
**Discussion and Conclusion**: To our knowledge, this is the first report discussing the relationship between MSCs and VM of cancer cells. This study establishes that MSCs in the bone microenvironment might promote the VM formations of Prostate cancer and points to SDF-1/CXCR4 axis and PI3K/Akt pathway as a potential target for therapeutic intervention. Better understanding of the mechanisms involved in this tumour stoma cell interaction may provide novel targets for the development of treatment strategies for prostate to bone metastasis.
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### 248
### ASSOCIATION OF 3q13.32 VARIANTS WITH HIP TROCHANTER AND INTERTROCHANTER BONE MINERAL DENSITY IDENTIFIED BY A GENOME-WIDE ASSOCIATION STUDY

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**Object**: Hip trochanter (TRO) and intertrochanter (INT) sub-regions have important clinical relevance to subtrochanteric and intertrochanteric fractures, but have rarely been studied by genome-wide association studies (GWASs).
**Subjects and Methods**: Aiming to identify genomic loci associated with BMD variation at TRO and INT regions, we performed a GWAS utilising the Framingham heart study (FHS, $N=6,912$) as a discovery sample, and utilised the Women's health initiative (WHI) African-American sub-sample ($N=845$), WHI Hispanic sub-sample ($N=446$), and Omaha osteoporosis study ($N=963$), for replication.
**Results**: Combining the evidence from both discovery and replication samples, we identified one novel locus, 3q13.32 (*rs1949542*, discovery p=6.16x10-8, replication p=2.86x10-4 for INT-BMD; discovery p=1.35x10-7, replication p=4.16x10-4

for TRO-BMD, *IGSF11*). We also replicated two loci 3p21 (*rs148725943*, discovery p=6.61x10-7, replication p=5.22x10-4 for TRO-BMD, *CTNNB1*) and 8q24 (*rs7839059*, discovery p=2.28x10-7, replication p=1.55x10-3 for TRO-BMD, *TNFRSF11B*) that were reported previously.
**Conclusion**: Our findings provide useful insights that enhance our understanding of bone development, osteoporosis, and fracture pathogenesis.
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### 253
### PHOTODYNAMIC THERAPY (PDT) TO ENHANCE HEALING OF FEMUR FRACTURES WITH A CRITICALLY SIZED DEFECT

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**Introduction**: The majority of long bone fractures heal successfully without complications; however fractures resulting from high impact trauma can result in delayed healing or non-union. Early intervention could decrease patient morbidity and reduce health care system costs. Photodynamic therapy (PDT) is a minimally invasive local treatment involving administration of a photosensitizer, which is activated by laser light leading to the production of singlet oxygen, which can induce apoptosis and/or necrosis of targeted cells and tissue and also influence immune responses. PDT treatment of metastatically involved vertebrae resulted in improved vertebral bone strength, stiffness and architecture, motivating studying PDT as an approach to augment bone healing. The aim of this study was to evaluate the ability of PDT treatment to enhance healing in fractures exhibiting critically size defects.
**Materials and Methods**: Femoral fractures with critically sized defects (6 mm) were generated in 30 adult female Sprague-Dawley rats (7 or 15 week survival). Under general anaesthesia an 8-hole PEEK plate was attached laterally to the femur. Using a Gigly saw, a bone piece was removed followed by closure of musculature and skin. Rats were randomly allocated to three groups: control, PDT applied either 1 day, or 7 days post fracture. A photosensitizer (Visudyne, Novartis, Canada) was injected (1mg/kg) followed 15 minutes later by light delivery (75J; 690 nm) using a 1 cm diffuser fibre placed parallel to the fracture. The rats were euthanized 7 or 15 weeks after induction of the fracture. μCT images of the femur at an isotropic 13.3 μm/voxel resolution (Inveon MicroCT, Siemens, Germany) were acquired and analysed (AmiraDev 5.2, FEI Visualization Science Group, USA). Thereafter, the bone was decalcified and processed for histology. Statistical analysis was performed using a 1-way ANOVA.
**Results**: All rats recovered well; however five animals were euthanized early due to plate displacement. The total bone volume (TV) evaluated from μCT images within the fracture gap did not show significant differences. In contrast, BMD (gHA/cm$^2$) trended toward higher values in the PDT treated groups compared to controls. The fracture gap measured on μCT images of the 7 week group demonstrated a trend toward smaller gaps in the PDT treated groups ($p = 0.0535$). A statistically significant ($p = 0.0085$) smaller gap is present in the PDT treated groups after 15 weeks. Histology of the control group showed more cartilage and woven bone formation in contrast to the PDT treated groups which exhibited more structured and mature bone.
**Discussion**: PDT treatment of rat femur fractures led to lower overall formation of bone, but the bone had higher density with a decrease in the size of the fracture gap. The increase in bone density in the PDT treated groups may suggest formation of better quality bone (vs. quantity of bone). Histologically, with more cartilage and woven bone present in the control group in contrast to more mature bone and in the PDT group, the fracture healing seems to follow a different pattern, which requires further investigation.
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### 258
### PATHOPHYSIOLOGY OF CHEMOTHERAPY-INDUCED DAMAGE OF BONE MARROW MICRO-VASCULATURE AND POTENTIAL PROTECTIVE EFFECTS OF FLAVONOIDS IN RATS

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**Introduction**: It has been widely shown that bone formation and remodelling would not occur unless there is already a properly established micro-vasculature. However, blood vessels can be damaged by extrinsic causes such as chemotherapeutic agents. Methotrexate (MTX) is an anti-metabolite chemo-agent widely used in treatment of many diseases including childhood leukaemia and inflammatory disorders. While previous studies showed that MTX can cause long-term skeletal side effects, whether and how it damages bone marrow micro-vasculature remains unclear. Using a rat model and endothelial cell-culture models, we addressed these questions. In addition, since we recently showed that the osteogenic, anti-oxidant, and anti-inflammatory flavonoid genistein can protect bone in MTX-treated rats, here we also investigated effects of genistein in the recovery of damaged blood vessels in rats treated with MTX. Furthermore, we also examined potential treatment effects of genistein and a related flavonoid, icariin, on viability and tube-formation ability of endothelial cells treated with MTX *in vitro*.
**Methods**: Animal studies: To study the effect of MTX on blood vessels, groups of male (6-week-old) Sprague—Dawley rats were subcutaneously injected with MTX (0.75mg/kg) once daily for 5 days and were sacrificed on day 1, 3, 6, 9, 11, and 14. To study the protective effects of genistein, in some MTX-treated rats, genistein was administrated by oral gavage (2 mg/100 g BW) for the whole period starting from day 0 until one day before kill (day 9). Treatment effects on number and sizes of bone marrow micro blood vessels were examined histologically in tibiae. MTT viability assay: Concentration-/time- dependent treatment effects of MTX (10nM-10μM) were examined on viability of cultured rat sinusoid endothelial cells (SECs) and effects of 24 hour treatment with MTX plus icariin/genistein (10nM-10μM) were also studied. Apoptosis detection by flow cytometry: SECs were treated with MTX (1μM/mL) for 24 and 48 hours and apoptosis was detected based on their cell surface Annexin-V expression. Tube formation assay: SECs were treated with/without MTX (1μM), icariin or genistein (100nM-10μM) and effects on angiogenesis were examined based on formation of tubes by SECs on Matrigel.
**Results**: Histological image analyses of H&E-stained tibial sections showed significant blood vessel damage in the bone marrow of rats on days 6 and 9 and significant but partial recovery on days 11 and 14 following the first MTX dose. Histology analyses suggested that genistein potentially attenuates MTX-induced blood vessel damage in the bone marrow. Examining any cytotoxic effect of MTX on endothelial cells, MTT assays showed that the viability of SECs was not affected after 24 hours of treatment with MTX (10nM-10μM). However, following 48 hour treatment, viability of SECs was reduced in a concentration-dependant manner. Flow cytometry analysis revealed that SECs underwent apoptosis following 48 hours (but not 24 hours) treatment with MTX (1μM). MTT assays also showed that neither genistein nor icariin treatment affected viability of SECs viability. Tube formation assays showed a reduced tube formation potential of SECs treated with MTX (1μM). Interestingly, icariin or genistein (10μM)-treated SECs showed enhanced tube formation and icariin or genistein treatment can prevent MTX-induced decrease in tube formation.
**Discussion and Conclusion**: Our *in vivo* and *in vitro* studies suggest that MTX causes blood vessel damage in the bone marrow, potentially by inducing apoptosis in endothelial cells and also interfering in the process of angiogenesis. Our *in vitro* tube formation assays showed that icariin and genistein might not only promote angiogenesis but possess some protective effect against MTX damage. Consistently, our *in vivo* studies also showed some positive effects of genistein treatment in reducing MTX-induced blood vessel damage in the bone marrow of rats.
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### 259
### INCREASED EZH2 COMBINED WITH DECREASED OSTEOBLASTOGENESIS IN LOCAL IRRADIATION INDUCED RAT BONE LOSS

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Radiation therapy is a common treatment for cancer patients. The adverse effects are the insufficiency fractures and bone loss. Epigenetic regulation plays an important role in the BMSCs differentiation. We reported here, the changes of local bone after a single-dose $^{137}C_S$ irradiation exposure in rats. The bone mineral density (BMD) of the femur and the trabecular bone volume in the tibia were significantly decreased at 12 weeks after irradiation. The micro-CT results showed that the tBMD, Tb.h, and Tb.N were also significantly reduced after 12 weeks of irradiation exposure. The ALP-positive OB.S/BS was decreased by 42.3% after 2 weeks irradiation, and decreased by 50.8% at the 12 weeks. In contrast to the decreased expression of Runx2 and BMP2, we found EZH2 expression was significantly increased after 2 weeks of single-dose $^{137}C_S$ irradiation in BMSCs. In conclusion, our results demonstrated that the single-dose $^{137}C_S$ irradiation induces the loss of BMD and bone micro-architecture deterioration in rat skeleton, as well as the increased expression of EZH2 and decrease of osteoblastogenesis after irradiation. The underlying mechanisms may be required to further investigate the relationship.
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### 261
### MYRICITRIN INHIBITS OSTEOCLASTOGENESIS

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Myricitrin is a botanical flavonol glycoside, extracted from leaves of *Myrica cerifera* and other plants. Abundant evidence supports myricitrin has anti-oxidative, anti-inflammatory, and neuroprotective effects. Osteoclastic bone resorption is